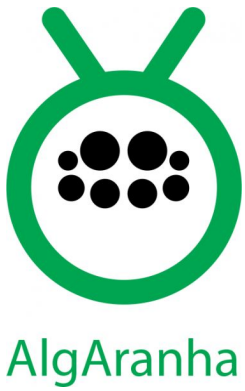
USER multimerization of synthetic MaSp1 DNA monomer



USER cloning is a seamless cloning technique based on uracil containing primers. A mix of enzymes which specifically remove a single strand downstream of an uracil base creates long overhangs, which anneal and can be transformed without ligation.

Summary

Amplifying/adapting the MaSp1 fragments for USER cloning:

- PCRs were not quite specific, but the primers ordered from Eton biosciences seemed to work (08/11). However, they were sent mistakenly without the ‘U’ base by the company. As we have to import those primers, we had to wait almost 2 months for the second batch to come and advance this part(20/09).

- With IDT primers with uracil, we had mixed succes depending on the template used and other reaction condictions. We were not able to optimize it, but there were bands of the right sizes. Importantly, the use of an “uracil compatible” polymerase is essential (home made X7 worked, Q5 high fidelity is not recommended).

Cloning reaction

- We tried to perform the cloning reaction directly with the PCR products (as the USER enzyme is stable in a wide variety of conditions) and got colonies.

- Pool of colonies was grown, miniprepped, concentrated and digested. Many bands were found, albeit way less mobile than the putative excised fragments. It was tempting to assume that they are uncut plasmids with varying insert size.

We were not able to verify the proper cloning and produce the parts before iGEM. This will be pursued as an after-competition effort.

----------08/11----------

PCR with USER primers for MaSp1 Type2 (1)

Brayan and Tiago

A previously standardized PCR (for amplification of IDT gBlock fragments) was done as the first PCR step for the USER multimerization of the “Lh MaSp1 Modified Type2” blocks.

The following pairs were used for the USER construction:

USER MaSp1 Type2 A (UMT2-A) - iGP0007 and iGP0010
USER MaSp1 Type2 B (UMT2-B) - iGP0009 and iGP0010
USER MaSp1 Type2 C (UMT2-C) - iGP0009 and iGP0008

USER Primers sequence:
https://docs.google.com/spreadsheets/d/1cTGOI98HkT89L4ywoOfZdqL1rTeQW4j6tYL2IJpl_b8/edit#gid=0

Master mix preparation for MaSp fragments (USER downstream applications):

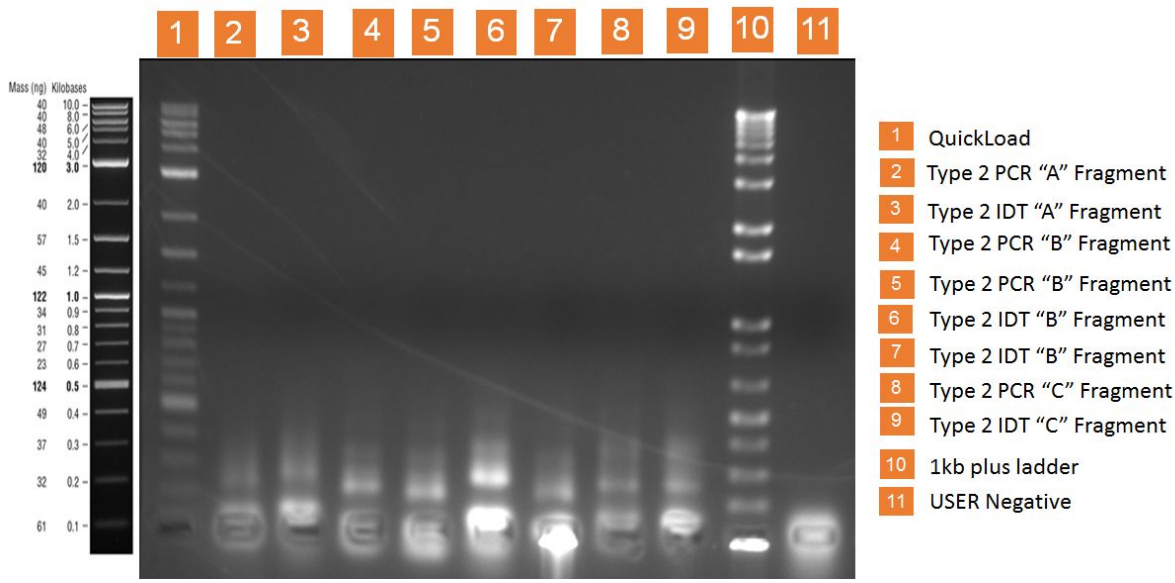
Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume “n” PCR reactions
Buffer GC	5X	X	2,5	n*2,5
dNTPs	10 mM	0,5 mM	0,625	n*
Primer forward*	10 uM	0,5 uM	0,625	n*
Primer reverse*	10 uM	0,5 uM	0,625	n*
Betaine	5 M	M	2,5	n*
X7 polymerase	100X		1,0	n*
DNA template (PCR product)	-	-	2,0	Each reaction needs the proper sequence
DEPC water			2,625	n*
			12,5	n*12,5

Cycle conditions:

Touch-down PCR (each cycle 63°C to 56°C + 25 cycles at 60°C)

	Temperature	Time	
Initial denaturation	98°C	1 min	
Rest of cycling	98°C	15 seg	25 additional cycles
	63°C-58°C (in steps of 1)	30 seg	
	72°C	2 min	
Final extension	72°C	5 min	
Hold	4°C	Hold	

USER Results



* Seems like USER primers are annealing elsewhere amplifying unspecific regions. It may be due to touch down PCR conditions that primarily aims to yield regardless the primers specificity. Also demonstrates that low temperature annealing (56 ~ 63°C) may not be the best for this reaction.

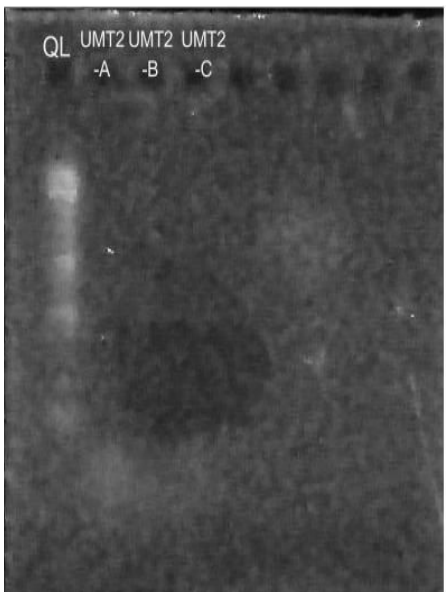
----------09/20----------

PCR with USER primers for MaSp1 Type2 (2)

Tiago

Using new primers, with uracil (https://drive.google.com/drive/folders/0B-0Ed5rht7A8dno5WGc1Ul9NWWc, IGP0031 - IGP0034) a 2step PCR was performed with NEB's Q5 High fideity 2x master mix. The template used was 4ng of MaSp1 type2 PCR product per 25 microliter reaction.

USER MaSp1 Type2 A (UMT2-A) - iGP0031 and iGP0034
USER MaSp1 Type2 B (UMT2-B) - iGP0033 and iGP0034
USER MaSp1 Type2 C (UMT2-C) - iGP0032 and iGP0033



Annealing/extension time was 20 seconds.

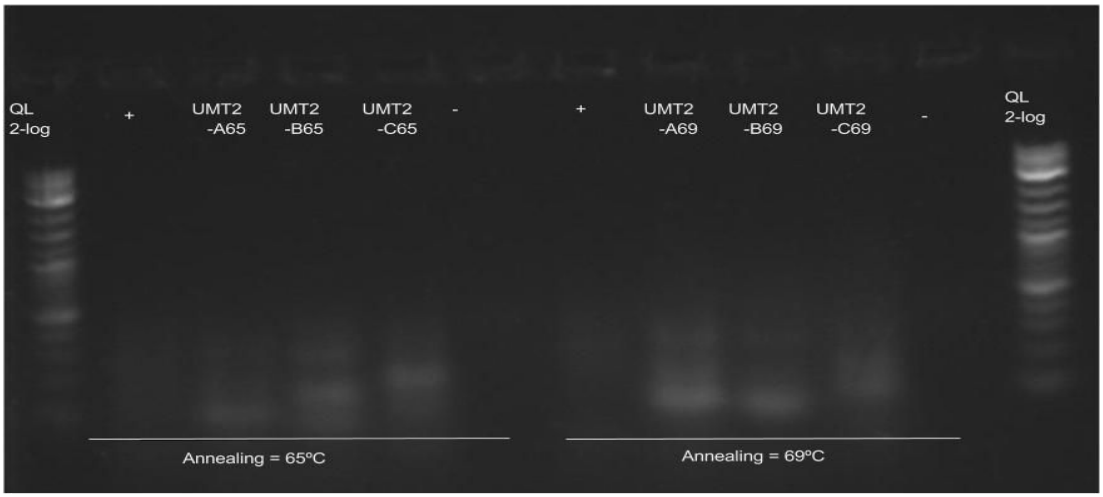
OBS: We didn`t realize that NEB does not recommend the use of Q5 polymerase for reactions with uracil containing primers. The PCR will be re-done with the homemade Pfu X7 polymerase.

PCR with USER primers for MaSp1 Type2 (3)

Tiago

Using primers with uracil, X7 home made polymerase and a PCR product as a template

USER MaSp1 Type2 A (UMT2-A) - iGP0031 and iGP0034
USER MaSp1 Type2 B (UMT2-B) - iGP0033 and iGP0034
USER MaSp1 Type2 C (UMT2-C) - iGP0032 and iGP0033



Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume "n" PCR reactions
Buffer GC	5X	X	5	n*5
dNTPs	10 mM	0.5 mM	1.5	n*
Primer forward*	10 uM	0.5 uM	1.25	n*
Primer reverse*	10 uM	0.5 uM	1.25	n*
Betaine	5 M	M	5	n*
Phusion polymerase	100X		2.0	n*
DNA template	-	-	1.0	Each reaction needs the proper sequence
DEPC water			to 25	n*
			25	n*25

----------09/22----------

PCR with USER primers for MaSp1Type2 (4)

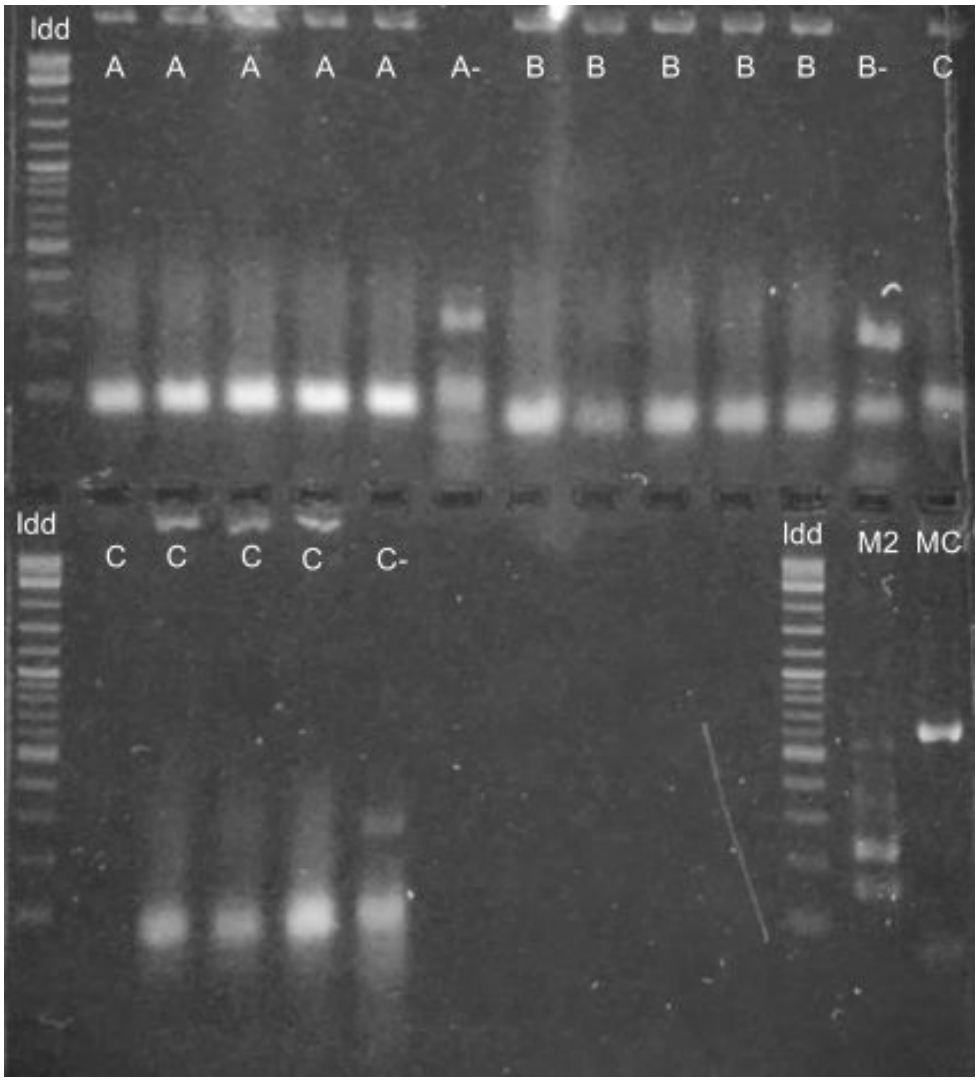
Tiago and Viviane

As we were not able to amplify it sucessfully, we tried again the USER PCR with the primers with uracil, X7 home made polymerase and, now, a Alkaline lysis of pJP22 + MaSp1 type2 cells as a template

USER MaSp1 Type2 A (UMT2-A) - iGP0031 and iGP0034
USER MaSp1 Type2 B (UMT2-B) - iGP0033 and iGP0034
USER MaSp1 Type2 C (UMT2-C) - iGP0032 and iGP0033

Temperatures for the gradient were 59, 60, 61, 63, 66 and the negative control was run at 66.

Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume "n" PCR reactions
Buffer GC	5X	X	5	n*5
dNTPs	10 mM	0.5 mM	1.5	n*
Primer forward*	10 uM	0.5 uM	1.25	n*
Primer reverse*	10 uM	0.5 uM	1.25	n*
Betaine	5 M	M	5	n*
Phusion polymerase	100X		2.0	n*
DNA template	-	-	1.0	Each reaction needs the proper sequence
DEPC water			to 25	n*
			25	n*25



The negative control showed a band, while all the others didn’t. Perhaps this was due to some kind of contamination and excess template in the reaction

----------09/29----------

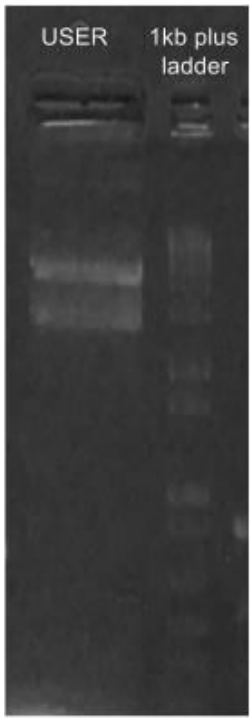
USER fusion of MaSp1Type2 - Incubation (1)

Tiago, João and Allan

The A, B and C parts of the USER fusion PCR were incubated in a thermocycler as follows:
1 microliter of USER enzyme (NEB)
1 microliter of USER cassete (from Stephen Mayfield's lab at UCSD)
1.5 microliters of UMT2-A69 PCR product (not purified)
1.5 microliters of UMT2-C65 PCR product (not purified)
5 microliters of UMT2-B65 PCR product (not purified)

15 minutes 37ºC
5 minutes 25ºC

All the volume was used to transform on-day electrocompetent cells (done by João).
The colonies were pooled for growth ON, miniprepped, concentrated in a vaccuum concentrator and digested, again ON with XhoI/BamHI. The



The electrophoresis indicates that the digestion was not successful, but possibly there are correct clones, as it appears to show bands of different sizes (corresponding to the inclusion of different numbers of MaSp1 Type2 parts in each vector.

PCR with USER primers for MaSp1Type2 (5)

Tiago

As we were not able to amplify it sucessfully, we tried onceagain the USER PCR with the primers with uracil, X7 home made polymerase and, now, a alkaline lysis and ethanol precipitation of pJP22 + MaSp1 type2 cells as a template BUT diluted 100 times.

USER MaSp1 Type2 A (UMT2-A) - iGP0031 and iGP0034
USER MaSp1 Type2 B (UMT2-B) - iGP0033 and iGP0034
USER MaSp1 Type2 C (UMT2-C) - iGP0032 and iGP0033

The temperature used for the annealing was 65.

Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume "n" PCR reactions
Buffer GC	5X	X	5	n*5
dNTPs	10 mM	0.5 mM	1.5	n*
Primer forward*	10 uM	0.5 uM	1.25	n*
Primer reverse*	10 uM	0.5 uM	1.25	n*
Betaine	5 M	M	5	n*
Phusion polymerase	100X		2.0	n*
DNA template	-	-	1.0	Each reaction needs the proper sequence
DEPC water			to 25	n*
			25	n*25

----------10/05----------

USER fusion of MaSp1Type2 - Incubation (2)

Tiago

The A, B and C parts of the USER fusion PCR were incubated in a thermocycler as follows:

1 microliter of USER enzyme (NEB)

1 microliter of USER cassete (from Stephen Mayfield's lab at UCSD)

1.5 microliters of UMT2-A69 PCR product (not purified)

1.5 microliters of UMT2-C65 PCR product (not purified)

5 microliters of UMT2-B65 PCR product (not purified)

15 minutes 37ºC

5 minutes 25ºC

This time, a negative control without USER enzyme (total 9 microliters) was also prepared. All the volume was used to transform on-day-made electrocompetent cells.